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Biased T-Cell Receptor δ Element Recombination in *scid* Thymocytes

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Thymocytes in mutant mice with severe combined immunodeficiency (*scid* thymocytes) show ongoing recombination of some T-cell receptor δ gene elements, generating signal joints quantitatively and qualitatively indistinguishable from those in wild-type fetal thymocytes. Excised Dδ2-Jδ1 and Dδ1-Dδ2 rearrangements are detectable at levels equivalent to or greater than those in thymocytes from wild-type mice on fetal day 15. Signal junctional modification, shown here to occur frequently in wild-type adult but not newborn excised Dδ2-Jδ1 junctions, can occur normally in adult *scid* thymocytes. Excised Dδ1-Dδ2 *scid* junctions, similar to wild-type thymocytes, include pseudonormal coding junctions as well as signal junctions. Inversional Dδ1-Dδ2 rearrangements, generating conventional hybrid junctions, are also reproducibly detectable in *scid* thymus DNA. These hybrids, unlike those reported for artificial recombination constructs, do not show extensive nucleotide loss. In contrast to the normal or high incidences of Dδ1-, Dδ2-, and Jδ1-associated signal junctions in *scid* thymocytes, Vδ1, Vγ3, and Vγ1.2 signal products are undetectable in *scid* thymocytes or are detectable at levels at least 10-fold lower than the levels in wild-type fetal thymocytes. These findings confirm biased T-cell receptor element recombination by V(D)J recombinase activity of nontransformed *scid* thymocytes and indicate that analysis of in vivo-mediated gene rearrangements is important for full understanding of how the *scid* mutation arrests lymphocyte development.

V(D)J recombinase activity mediates somatic recombination of antigen receptor gene segments in developing B and T lymphocytes. The standard products of this recombination process are coding junctions and their reciprocal signal junctions. These junctions are formed by paired ligation events following cleavage at the border of coding and conserved recombination signal sequence segments (reviewed in references 2, 27, and 29). Recombination consensus signal sequences (RSS), which confer recognition for lymphoid-specific recombinase activity, consist of strongly conserved heptamer and moderately conserved nonamer sequences, separated by either 12 or 23 bases (18). In deletional recombination, coding junctions are retained in the genome and signal junctions are chromosomally excised. Whereas coding junctions are commonly modified by base loss and addition, signal junction formation is usually precise (27, 29). Nonstandard resolution of coding and signal ends, generating hybrid junctions in which the signal sequence of one segment is joined to the coding sequence of another segment, has also been reported (28, 33).

In mice homozygous for the *scid* mutation (5), V(D)J recombinase activity is severely impaired (30, 42), resulting in the failure to generate both T and B lymphocytes. Definition of V(D)J recombinase impairment by the *scid* mutation has been derived largely from studies of transformed *scid* lymphoid cell lines. In Abelson murine leukemia virus-transformed *scid* bone marrow cell lines, recombined immunoglobulin heavy-chain genes show gross deletions (>1 kb) of coding sequence (16, 25, 32). Spontaneous *scid* thymic lymphomas contain similar gross deletions for T-cell receptor (TCR) γ and TCR β genes (41, 42). Transfection of artificial recombination substrates into *scid* transformed lymphoid cell lines further indicated severe impairment of *scid* V(D)J recombinase activity; coding junction formation is undetectable or extremely rare (15, 30). Signal junction formation of transfected recombination substrates occurs at a normal frequency, but signal joints commonly show excessive nucleotide loss (30). Hybrid junctions formed in *scid* lymphoid cell lines also show excessive base loss (30).

Recent studies of TCR gene rearrangement in nontransformed *scid* thymocytes have indicated less severe qualitative and quantitative impairment of coding junction formation (7). Developmentally arrested *scid* thymocytes invariably show rearrangement at the TCR δ locus but not at TCR α, β, and γ loci. These findings supported evidence that the TCR δ locus is the earliest TCR gene to undergo ordered recombination (10) and suggested that only the earliest V(D)J recombination events are completed. *scid* thymocytes show rearrangement of specific TCR δ elements, notably Dδ1, Dδ2, and Jδ1; Vδ1 element-associated coding junction formation, believed to occur concurrently with Dδ2 and Jδ1 recombination (10), has not been detected (7). The basis of selective TCR δ element participation in coding joint formation in *scid* thymocytes has been unclear. *scid* thymocytes may mirror the actual ordered sequence of rearrangement for at least one wave of thymic ontogeny; alternatively, the *scid* mutation may bias toward initiation and resolution of recombination complexes for specific TCR gene elements.

Notably, sequence analysis of cloned *scid* thymocyte Dδ2-Jδ1 coding junctions of an individual thymus from a mouse with severe combined immunodeficiency showed that rearrangements were clonally heterogeneous and that most were indistinguishable from normal coding junctions (7). The apparently normal structure and high incidence of TCR δ coding junctions in nontransformed *scid* thymocytes indicated that Abelson murine leukemia virus-transformed *scid* bone marrow cells do not accurately represent how the *scid* mutation manifests in vivo. The present studies were designed to further address how the *scid* mutation manifests in

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nontransformed, developing thymocytes. Since the *scid* defect has not been shown to impair the incidence of signal joint formation, we have further analyzed the incidence and structure of excised signal junctions to assess the frequency of initiation and completion of V(D)J recombination events for specific TCR loci and elements. We have used a sensitive polymerase chain reaction (PCR) assay to qualitatively and semiquantitatively analyze the formation of specific TCR δ- and TCR γ-associated coding, signal, and hybrid junctions. The present studies show that *scid* thymocytes mediate ongoing recombination of TCR Dδ1, Dδ2, and Jδ1 elements and that signal junctions formed are quantitatively and qualitatively equivalent to those of wild-type fetal thymocytes. TCR δ signal junction modification, detected in wild-type thymocytes from adult but not newborn mice, occurs normally in *scid* thymocytes. In contrast, recombination of Vδ1 and Vγ elements is not routinely detectable.

MATERIALS AND METHODS

Mice. C.B.-17 (an immunoglobulin heavy-chain congenic strain of BALB/c) wild-type and *scid* mice were purchased from specific-pathogen-free breeder stocks from Taconic Laboratory Animals and Services, Germantown, N.Y. They were bred and maintained in specific-pathogen-free animal rooms in microisolator cages with autoclaved food and water. Newborn mice were obtained within 48 h of parturition.

PCR amplification. PCR primers were based on published sequence and were purchased from National Biosciences, Plymouth, Minn. Sequences for reverse-oriented Vγ3-Jγ1 primers (p11 and p12) were obtained from published primer sequences (1). Primer sequences for β_2-microglobulin are as follows: 5′, AGCATGACAGTATGGCCGA; 3′, GCTGAT CACATGTCTCGATC. TCR primers, with internal (int) primers used in secondary PCR noted, are as follows: p1, CAGTGGGTATGGCAGAGGGT; p1-int, GGGATCCGGT GATGGCAAAATGCCAA; p2, ATTCTAGACAGAGGC CAGCAAGTGGA; p3, CTGAATCCGTCACTGTTAGTC CGCT; p3-int, ATCTGCAGTCCGCTTGATCAATATTGA GGA; p4, CTTAAGTACCCAGGTCAAGTCT; p4-int, AAA AGATCTGGCCTGAACTAACTGCCA; p6, CTGTACCTC CTGTAAGCTAACCCAT; p6-int, GTAGATCTGGATGAG TAACACATGCTGT; p7, CAGGACGGTCTCATCTCAGA TGT; p8, CTCGACATTCAGAAGGCAACA; p9, GGAAG CTTTCCAGATGGAGACTCCT; p10, TTCATCACTGGA ATAAAGCAG; p11, CAGGTTCCTCCAGATGCTGAGA; p12, TGCAATGACTTTCTAAGGAACC; p13, TTCTGGGT GCAGGAGACAGT; p14, GGGGGGGATCCAAGAGGAT GTGGGTG; p15, CATGTAATGGCCTTCT. In genomic DNA PCR amplification (38), reactions were carried out in 50-μl volumes in the presence of 100 μM (each) dATP, dCTP, dGTP, and dTTP; 1 μM each oligonucleotide primer; 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 1.5 mM $MgCl_2$; and 0.01% gelatin. Primary PCR was carried out with DNA from thymocyte lysates prepared by the method of Schlissel and Baltimore (40). The genome equivalent of 2×10^4 cells per reaction was used as a template. Reactions were done for an appropriate number of cycles predetermined to be within the exponential range of amplification for each primer set used. For cloning, secondary PCR was carried out with 2 μl of the primary PCR reactant mixture and one primer (p-int) internal to the one used for primary PCR. Cycling was carried out in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, Conn.) by using the following protocol: 2 cycles at 94°C for 3 min, 50 to 63°C for 1 min, and 72°C for 1 min; and then 19 to 33 cycles at 95°C for 1 min, 50 to 63°C for 1 min, and 72°C for 1 min. For template titration analysis, serial dilutions (10^4, 10^3, and 10^2) of each template were used. For amplification of the β_2-microglobulin gene in titration experiments, the cycle number (21 cycles) was predetermined to ensure semiquantitative analysis over the template dilution range.

Analysis of DNA PCR amplification. PCR products of genomic DNA amplification were analyzed on 1% agarose gels, Southern blotted, and hybridized as described previously (7). Probes were labeled by the random-prime method (11). Labeled probe hybridization on blots was quantitated by using a Betascope (Betagen, Waltham, Mass.). Quantitative values for TCR gene rearrangement products for a given template were derived by normalization against activity quantitated for parallel-amplified β_2-microglobulin.

Cloning and sequencing. Secondary PCR products containing TCR δ rearrangements were cloned directly by using the TA cloning kit as specified by the manufacturer (Invitrogen, San Diego, Calif.). Transformants were screened by colony hybridization, as described previously (9). Double-stranded plasmid DNA was generated and sequenced by the dideoxy-chain termination method (39) with appropriate primers and the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). Labeled DNA was run on Long Ranger (AT Biochem, Malvern, Pa.) sequencing gels.

Hybridization probes. 5′Dδ2 and DJδ probes were derived from pJδ7, a 7.5-kb *Eco*RI fragment spanning the sequence 5′ of Dδ2 to 3′ of Jδ1, as described previously (7). 5′Dδ2 is a 2-kb *Pst*I fragment, and DJδ is a 1-kb PCR-amplified fragment that spans the region between Dδ2 and Jδ1. Probe Vδ1 (pVM9), a 410-bp *Xba*I-*Eco*RV fragment (10), was kindly provided by Y. Chien. Probe Vγ1.2 was derived from the cDNA clone p8/10-2γ1.1 (20). The following probes were produced by PCR amplification with appropriate primers: 5′-3′-Vγ3, 5′-3′-Jγ1, and β_2-microglobulin.

Restriction and Southern blot analysis. Genomic DNA was prepared by the method of Blin and Stafford (4), and digested DNA (10 μg) was analyzed by Southern analysis as described previously (7).

RESULTS

Ongoing recombination of TCR Dδ1, Dδ2, and Jδ1 in *scid* thymocytes. Dδ1-, Dδ2-, and Jδ1-associated coding junctions are routinely detectable in *scid* thymus DNA preparations (7; data not shown), and PCR-amplified Dδ2-Jδ1 coding joints from an individual thymus from a mouse with severe combined immunodeficiency are clonally variable and indistinguishable from the wild type (7). To confirm that these TCR δ elements undergo ongoing recombination, we analyzed independent *scid* thymic lysates by PCR, using primers designed to amplify excised, circular signal junctions. Results of PCR amplification of Dδ2-Jδ1 and Dδ1-Dδ2 excision products are shown in Fig. 1. The TCR δ primers used and their relative orientations are shown; primers with the opposite orientation on the germ line could amplify circular excision products. For each template, parallel amplification of the single-copy β_2-microglobulin gene provided an internal normalizing control. Individual *scid* thymic lysates, derived from newborn or 4-week-old mice, reproducibly showed amplification of excised, circular Dδ2-Jδ1 and Dδ1-Dδ2 reciprocal junctions. Wild-type thymic lysates from newborn mice or 15-day-old fetuses also showed amplification of TCR δ excision products. Since *scid* thymocytes show surface marker expression equivalent to normal fetal

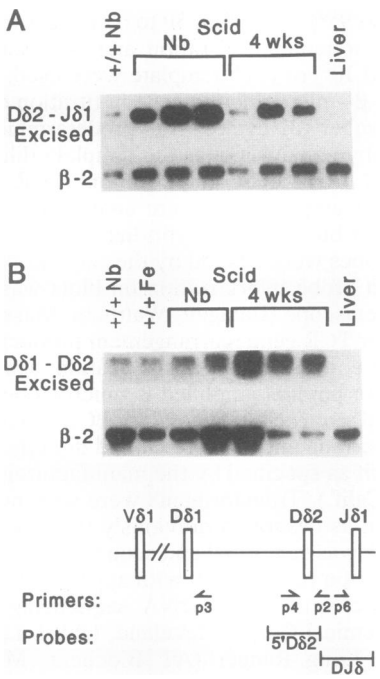

FIG. 1. *scid* thymocytes mediate ongoing recombination of TCR Dδ1, Dδ2, and Jδ1 elements, generating Dδ2-Jδ1 and Dδ1-Dδ2 signal junctions. PCR-amplified circular excision products of Dδ2-Jδ1 (A) and Dδ1-Dδ2 (B) rearrangements, with parallel amplification of the β_2-microglobulin single-copy gene, from individual thymic lysates of wild-type mice (+/+) and mice with severe combined immunodeficiency (Scid), aged fetal day 15 (Fe), newborn (Nb), or 4 weeks. (A) Dδ2-Jδ1 excision products were amplified with primers p2 and p6, generating a 372-bp fragment, detectable by hybridization with the DJδ probe. (B) Excised Dδ1-Dδ2 rearrangements were amplified with primers p3 and p4. Unmodified signal junctions would result in a 425-bp amplification fragment, detectable by hybridization with the 5′Dδ2 probe.

day 15 thymocytes (8, 14), we compared the incidences of TCR δ rearrangements in individual *scid* thymocyte samples with those in wild-type fetal thymocytes. The estimated incidence of Dδ2-Jδ1 and Dδ1-Dδ2 excised (signal) junction formation in all *scid* thymic lysates tested was reproducibly equal to or higher (2- to 10-fold) than that of wild-type fetal thymus.

***scid* TCR δ signal junctions are indistinguishable from wild type and show age-associated junctional modification.** Cloning and sequence analysis of PCR-amplified Dδ2-Jδ1 signal junctions from *scid* thymocytes showed them to be indistinguishable from age-matched wild-type thymocytes (Fig. 2A). All cloned Dδ2-Jδ1 signal junctions from wild-type thymic lysates from newborn mice showed precise heptamer-heptamer fusion, with no nucleotide insertion or excision. Of 11 *scid* thymic junctions from newborn mice, 10 were also precise. Notably, however, a high percentage of both wild-type and *scid* adult thymus-derived signal junctions showed junctional modification. Three of six sequenced wild-type adult thymocyte clones showed nonprecise junctions, characterized by nontemplated nucleotide insertion. One clone also showed excision of the Dδ2-associated heptamer. Heptamer excision, previously reported as characteristic of *scid*- but not wild-type-mediated signal junction formation on artificial substrates (30), has also been detected at a low frequency in other wild-type TCR δ junctions (9). *scid* thymic Dδ2-Jδ1 signal junctions from adult mice also showed frequent junctional modification. Of 29 sequenced clones of an individual *scid* thymic lysate from a 4-week-old mouse, 14 showed nontemplated junctional insertion; 2 of 29 also showed nucleotide loss (2 to 3 bp). The sequence data also indicate the clonal heterogeneity of signal junctions from the DNA of an individual thymus from a mouse with severe combined immunodeficiency. The generality of age-associated modification of Dδ2-Jδ1 signal junctions was indicated by restriction analysis of PCR-amplified rearrangements with the enzyme *Apa*LI, which cleaves the consensus site generated by perfect heptamer-heptamer fusion (GTGCAC); a higher incidence of *Apa*LI restriction was detected for fetal and newborn thymic amplified signal junctions (70 to 95%) than for adult thymic amplified junctions (40 to 50%) (data not shown).

PCR-amplified Dδ1-Dδ2 excision junctions also were cloned and sequenced from *scid* thymocyte DNA (Fig. 2B). The predicted excision product for deletional Dδ1-Dδ2 rearrangement is a signal junction formed by fusion of recombination signal sequence heptamers 3′ of Dδ1 and 5′ of Dδ2. As reported previously, however, Dδ1-Dδ2 recombination in wild-type thymocytes generates a high incidence of nonstandard products, probably as a result of participation of two short (11- to 16-bp) coding elements, each flanked by two recombination signal sequences (9). These nonstandard products include pseudonormal rearrangements in which external signal sequences (5′Dδ1 and 3′Dδ2) fuse, generating signal joints which are retained on the chromosome. Excised rearrangements thus include coding joints. They also include hybrid junctions, and some rearrangements show evidence for open-shut or oligonucleotide capture events (9). *scid* thymocyte Dδ1-Dδ2 excision products also showed pseudonormal coding joints (Fig. 2B). One adult *scid* thymus-derived clone contained both Dδ1 and Dδ2 coding segments, with limited base loss and insertion of a single base. One newborn *scid* thymus-derived clone probably represents an additional coding joint in which homologous overlapping nucleotides (ATC) of Dδ2 and Dδ1 participated in junctional resolution (although we cannot rule out its alternative derivation by hybrid joint formation). Other *scid* clones included signal junctions and additional nonstandard products, similar to those seen for wild-type thymus. Dδ1-Dδ2 excised signal joints characteristically retained CA nucleotides, presumably derived from Dδ1 coding sequence, as previously reported for wild-type junctions (9, 43).

***scid* thymocytes form Dδ1-Dδ2 inversional hybrid junctions.** The sequenced Dδ1-Dδ2 amplified clones shown above were products of deletional recombination, in which cleavage occurred at RSS either external or internal to Dδ1 and Dδ2 elements. Inversional recombination could also occur between these elements; if cleavage occurred at either internal or external RSS, in concordance with the 12/23 spacer rule, the expected products would be paired (reciprocal) hybrid junctions, as shown in Fig. 3A. On the basis of studies with artificial recombination substrates, reciprocal hybrids were predicted to occur at a low incidence in normal cells (28). To determine whether Dδ1-Dδ2 reciprocal hybrids were generated in wild-type or *scid* thymocytes in vivo, we amplified template DNA of individual thymic lysates by PCR with primers oriented in the same direction. Amplification should result only if inversional recombination has occurred. As shown in Fig. 3B, all *scid* thymic lysates, but neither wild-type thymic lysate, showed amplification of a fragment of the predicted size (259 to 265 bp) for an inversional hybrid rearrangement.

GERMLINE SEQUENCE:

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Dδ1    TGTTTTTGTACGGCTGTGTTTCACTGTGGTGGCATATCACACAGGTTGAAGT
Dδ2:   GGTTTTTGCAAAGCTCTGTAGCACCGTGATCGGAGGGATACGAGCACAGTGTTGCAAACCC
Jδ1:         ...AATGGCCTCAGTAGCTGTGCTACCGACAAACTCGTCTTTGGACAAGGAAACCC...
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A. Dδ2-Jδ1 EXCISION PRODUCTS:

		Jδ1	(N)	Dδ2	# Clones
+/+	newborn	AATGGCCTCAGTAGCTGTG		CACAGTGTTGCAAACCC	9/9
scid	newborn	AATGGCCTCAGTAGCTGTG		CACAGTGTTGCAAACCC	10/11
		AATGGCCTCAGTAGCTGTG		AGTGTTGCAAACCC	1/11
+/+	adult	AATGGCCTCAGTAGCTGTG		CACAGTGTTGCAAACCC	3/6
		AATGGCCTCAGTAGCTGTG	AGAA	CACAGTGTTGCAAACCC	1/6
		AATGGCCTCAGTAGCTGTG	AGAGAG	CACAGTGTTGCAAACCC	1/6
		AATGGCCTCAGTAGCTGTG	GAATGGG	TTGCAAACCC	1/6
scid	adult	AATGGCCTCAGTAGCTGTG		CACAGTGTTGCAAACCC	15/29
		AATGGCCTCAGTAGCTGTG	CC	CACAGTGTTGCAAACCC	4/29
		AATGGCCTCAGTAGCTGTG	GA	CACAGTGTTGCAAACCC	1/29
		AATGGCCTCAGTAGCTGTG	C	CACAGTGTTGCAAACCC	1/29
		AATGGCCTCAGTAGCTGTG	G	AGTGTTGCAAACCC	1/29
		AATGGCCTCAGTAGCTGTG	AT	CACAGTGTTGCAAACCC	1/29
		AATGGCCTCAGTAGCTGTG	GT	CACAGTGTTGCAAACCC	1/29
		AATGGCCTCAGTAGCTG	CC	CACAGTGTTGCAAACCC	1/29
		AATGGCCTCAGTAGCTGTG	CTCC	CACAGTGTTGCAAACCC	2/29
		AATGGCCTCAGTAGCTGTG	CGG	CACAGTGTTGCAAACCC	1/29
		AATGGCCTCAGTAGCTGTG	GGGT	CACAGTGTTGCAAACCC	1/29

B. Dδ1-Dδ2 EXCISION PRODUCTS:

	Dδ2		(N)	Dδ1	# Clones
scid newborn	TCTGTAGCACCGTG			CACACAGGTTGAAGT	4/8
	TCTGTAGCACCGTG			ATCACACAGGTTGAAGT	1/8
	TCTGTAGCACCGTG	AGGG		CACACAGGTTGAAGT	1/8
	TCTGTAGCACCGTG		T	ACACAGGTTGAAGT	1/8
	TCTGTAGCACCGTG		CCC	CACACAGGTTGAAGT	1/8
scid adult	TCTGTAGCACCGTG			CACACAGGTTGAAGT	3/7
	TCTGTAGCACCGTGATCGGAGGGATAC		C	GCATATCACACAGGTTGAAGT	1/7
	TCTGTAGCACCGTGA		GA	CACACAGGTTGAAGT	1/7
	TCTGTACCACCGTG		T	TCACACAGGTTGAAGT	1/7
	TCTGTAGCACCGTG		GG	TCACACAGGTTGAAGT	1/7

FIG. 2. Junctional sequences for *scid* Dδ2-Jδ1 and Dδ1-Dδ2 excised rearrangements are indistinguishable from wild-type sequences. Germ line sequences for Dδ1, Dδ2, and Jδ1 (partial) are shown, with coding sequences in boldface type and flanking signal heptamer and nonamer sequences underlined. (A) Junctional sequences of PCR-amplified Dδ2-Jδ1 signal junctions from wild-type (+/+) and *scid* newborn and adult (4 to 8 weeks) thymic lysates, with primers p2 and p6. Sequences from newborn mice derive from pools of two thymuses each; sequences from adult mice derive from single individual mice. Junctional insertions are indicated as N. (B) *scid* thymic PCR-amplified, cloned Dδ1-Dδ2 circular excision products show conventional signal junctions, excised coding junctions, and hybrid junctions. Primers p3 and p4 were used for amplification. Junctional insertions are indicated as N.

Secondary PCR was done with amplified DNA of an individual *scid* newborn thymus template (G), and amplified DNA was cloned and sequenced (Fig. 3C). As illustrated in Fig. 3A, cleavage at alternate sites (rows a and b), both of which obey the 12/23 spacer rule, would yield similar products, differing only in the positions of the Dδ elements themselves. Cloned *scid* inversional rearrangements showed both types of product, in which the 5′Dδ1-5′Dδ2 hybrids retained either the Dδ1 (4/6) or Dδ2 (1/6) coding element. Base loss and addition were minimal, with two Dδ1-containing hybrids (G3, of identical sequence) containing a single nucleotide insertion. Base loss occurred only at coding ends and was not excessive (2, 3, or 8 bp). An additional clone (G2) lacked any Dδ coding sequence and had lost four Dδ1-associated heptamer bases. We cannot rule out the possibility that more than two recombinase-mediated cuts, as shown in Fig. 3A, occurred. In this case, excision of a Dδ coding segment would result in generation of a signal joint. It is notable, however, that wild-type reciprocal hybrid junctions formed on artificial substrates also showed large deletions (up to 28 bp) of coding flank sequence and loss of signal heptamer bases (28). The expected reciprocal product of a 5′Dδ1-5′Dδ2 inversional hybrid would be a 3′Dδ1-3′Dδ2 hybrid. These were also detected in *scid* thymic lysates by amplification with the appropriate primers (data not shown).

***scid* thymocytes do not show ongoing rearrangement of Vδ1.** Vδ1-associated rearrangements were undetectable or quantitatively reduced relative to the situation for wild-type thymocytes (Fig. 4A). In contrast to Dδ1-Dδ2- and Dδ2-Jδ1-associated rearrangements, Vδ1-Dδ2 coding junctions were rarely detectable, and when they were detectable they occurred at levels at least 10-fold lower than those of wild-type fetal thymus DNA. Concordant with these results, Vδ1-Dδ2 signal junctions were not reproducibly amplified from *scid* lysates. These signal products were more frequently amplified from newborn *scid* thymic lysates than from 4-week-old *scid* thymic lysates. When detectable, they occurred at levels generally 10-fold lower than in the wild-type fetal thymus.

The relative quantitative differences in recombination of Dδ2-Jδ1 and Vδ1-Dδ2 elements in *scid* and wild-type thymocytes were more specifically examined by PCR amplification of titrated DNA templates, as shown in Fig. 4B. Excised circular Dδ2-Jδ1 and Vδ1-Dδ2 signal junctions, and the β_2-microglobulin single-copy control gene were amplified in parallel from titrated template (10^4, 10^3, and 10^2 genomes) of

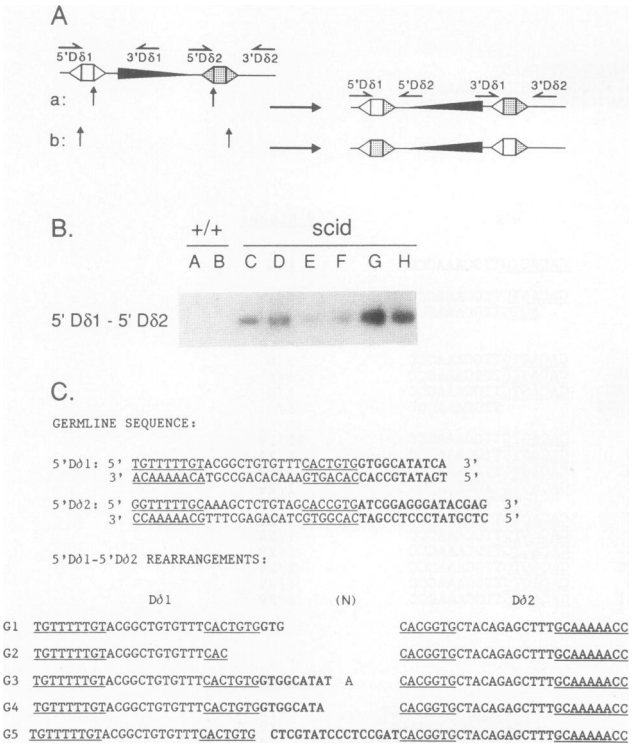

FIG. 3. Dδ1-Dδ2 inversional recombination generates conventional hybrid junctions in *scid* thymocytes. (A) Schematic of inversional Dδ1-Dδ2 recombination and predicted products. The black elongated triangle indicates altered orientation conferred by inversional recombination. Small rectangles indicate Dδ coding segments flanked on either side by consensus signal sequences (small triangles). Germ line 5′ signal sites contain 12-bp spacer segments, and 3′ signal sites contain 23-bp spaces. Alternative paired cuts which would obey the 12/23 spacer rule are shown. (B) PCR amplification of inversional recombination with primers to 5′Dδ1 (p1) and 5′Dδ2 (p4), generating products within the predicted size range (259 to 265 bp). Results, detected by Southern analysis with the 5′Dδ2 probe, are shown, derived from independent thymic lysates, as follows: lane A, wild-type (+/+) newborn; lane B, +/+ fetal; lanes C to E, *scid* adult; lanes F to H, *scid* newborn. (C) Cloned and sequenced *scid* thymic 5′Dδ1-5′Dδ2 inversional rearrangements from sample G. Germ line sequence shows the 5′ RSS for Dδ1 and Dδ2 elements, with both heptamer and nonamer sequences underlined. The Dδ coding sequence is shown in boldface type. Both coding and noncoding strands are shown to facilitate analysis of sequences (below) generated by inversional rearrangement. Sequence G3 was present in duplicate clones, with a single-base insertion (designated N).

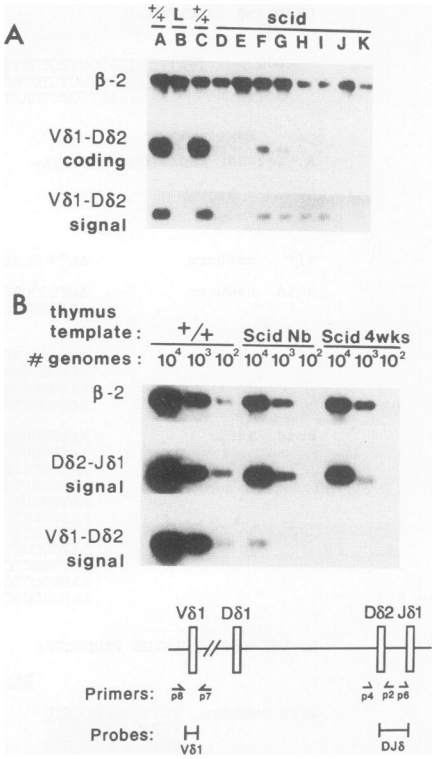

FIG. 4. Vδ1-Dδ2 gene rearrangement is rare in *scid* thymocytes. (A) PCR-amplified coding junctions and excised signal junctions for Vδ1-Dδ2 rearrangement, with parallel amplification of the β_2-microglobulin single-copy gene control (β-2), from individual thymic lysates of wild-type mice and mice with severe combined immunodeficiency. Coding junctions were amplified with primers p8 and p2, generating a 249-bp product; signal junctions were amplified with primers p7 and p4, generating a 289-bp product. Lysates in individual lanes are as follows: A, wild-type newborn thymus; B, *scid* liver (germ line control); C, wild-type fetal day 15 thymus; D to G, *scid* newborn thymus; H to K, *scid* 4-week thymus. (B) Titration of selected templates (wild-type fetal day 15 thymus, *scid* newborn thymus, and *scid* 4-week-old thymus) for genome equivalents of 10^4, 10^3, and 10^2, for PCR amplification of Dδ2-Jδ1 (p2 and p6) and Vδ1-Dδ2 (p7 and p4) signal joints. Results for the parallel amplified β_2-microglobulin single-copy gene (β-2) are also shown. Hybridization probes used were Vδ1, 5′Dδ2, and DJ8 for detection of amplified coding and signal junctions.

wild-type fetal thymus and representative *scid* thymic lysates, one each from mice which were newborn or 4 weeks of age. Dδ2-Jδ1 excision circles were amplifiable for all template dilutions of the *scid* thymic lysates as well as the wild-type fetal thymic lysate. In contrast, Vδ1-Dδ2 excision circles, amplified for all template dilutions of the wild-type thymic lysate, were detectable only from amplification of the most concentrated *scid* thymic lysate from newborn mice (10^4 genomes) and not at all from the *scid* thymic lysate from 4-week-old mice. Thus, within individual *scid* thymic lysates, Dδ2-Jδ1 excision products were quantitatively equivalent to (or higher than) those from wild-type fetal thymic lysate, whereas Vδ1-Dδ2 excision products were 1 or 2 orders of magnitude lower.

Another indicator of initiated TCR element recombination in *scid* thymocytes is the presence of cleaved, unresolved coding-end intermediates (36). Southern analysis of restricted *scid* thymocyte DNA with probes to Dδ2 and Jδ1 routinely detects non-germ line fragments unique to *scid* thymus (7). Non-germ line Jδ1 hybridizing *Eco*RI fragments of *scid* thymus DNA are shown in Fig. 5. Two of these restriction fragments (~4.0 and 4.9 kb) were recently identified as cleaved, unresolved coding-end intermediates generated by cleavage at RSS 5′ of Dδ2 and Jδ1 segments, which have been modified by hairpin formation (36). If *scid* thymocytes generate similar cleaved, unresolved Vδ1 coding intermediates, Southern analysis of *Eco*RI-restricted *scid* thymus DNA with a Vδ1-specific probe should detect, in addition to a germ line band of 7.2 kb, an additional fragment of ~4.8 kb. A duplicate sample of *Eco*RI-restricted *scid* thymus DNA, probed with a Vδ1-specific probe, showed no detectable non-germ line band which would correspond to a coding intermediate.

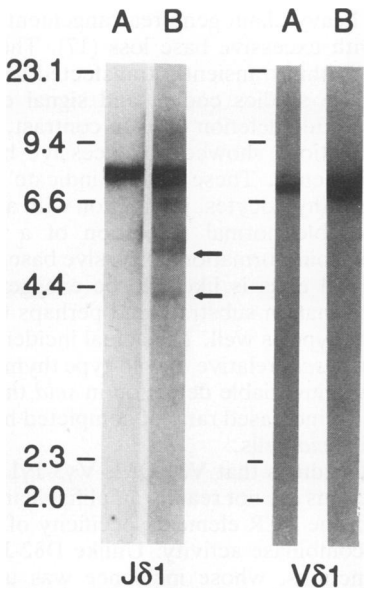

FIG. 5. *scid* thymus DNA shows the germ line Vδ1 sequence only. Southern analysis of *Eco*RI-restricted DNA of liver (germ line control; lanes A) and *scid* thymus (lanes B) DNA with probes to Jδ1 and Vδ1. Liver DNA shows germ line bands. *scid* thymus DNA shows cleaved coding intermediates for 5′Jδ1 (4.0 kb) and 5′Dδ2 (4.9 kb) but not for Vδ1. DNA size markers are indicated.

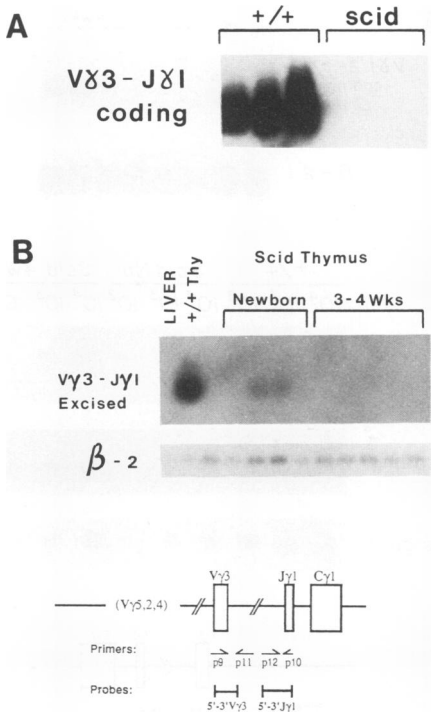

FIG. 6. Vγ3-Jγ1 rearrangement is rare in *scid* thymocytes. (A) PCR-amplified Vγ3-Jγ coding junctions, amplified with primers p9 and p10, from three individual wild-type newborn thymic lysates and three individual *scid* thymic lysates (one from a newborn mouse and two from 4-week-old mice). The hybridization probe used was 5′-3′Vγ3, and the unmodified amplified product is 418 bp. (B) PCR-amplified Vγ3-Jγ1 excised signal junctions (165 bp), amplified with primers p11 and p12, with parallel amplification of β_2-microglobulin (β-2). Results shown, hybridized with the 5′-3′Jγ1 probe, are from the following templates: *scid* liver, wild-type newborn thymus, five *scid* lysates from newborn mice, and five *scid* lysates from 3- to 4-week-old mice.

Recombinogenic inactivity of the TCR γ locus in *scid* thymocytes. Others have reported TCR γ rearrangements cloned from pooled *scid* thymus DNA preparations and have suggested that ongoing recombination of TCR γ, as well as TCR δ, loci may occur in *scid* thymocytes (23). To address this possibility, we did semiquantitative PCR analysis of specific TCR γ elements with individual *scid* thymic lysates as templates (Fig. 6 and 7). We first examined PCR amplification of coding and excised signal products for TCR Vγ3-Jγ1 rearrangements, which are reported to occur in the earliest stages of thymic ontogeny (13), generating the first wave of functional TCR γ/δ receptor-bearing cells. As shown in Fig. 6A, three independent *scid* thymic lysates failed to generate any detectable coding junctions, despite abundant amplification in wild-type thymus lysates. Use of reverse-oriented primers to amplify excised signal rearrangements (Fig. 6B) showed that only 2 of 10 *scid* lysates tested, both from *scid* thymic templates from newborn mice, generated a faintly detectable amplification signal. Betagen analysis indicated that this signal was at least 10-fold lower than the signal for the wild-type template.

We similarly determined the incidence of Vγ1.2-Jγ2 gene rearrangement. Coding and signal products of these elements were more reproducibly detectable from *scid* thymic lysates, notably the lysates from the older (4-week-old) mice (Fig. 7A). PCR titration analysis of TCR Vγ1.2-Jγ1 coding and signal junctions, relative to the wild-type fetal thymus template, however, showed that levels in *scid* lysates were generally lower by 1 to 2 orders of magnitude (Fig. 7A).

DISCUSSION

The present studies confirm that developing nontransformed *scid* thymocytes initiate V(D)J recombination, selectively recombining specific TCR δ elements. Previous studies indicated a high incidence of Dδ1, Dδ2, and Jδ1 coding joint formation (7), and a recent report has identified cleaved coding intermediates for Dδ2 and Jδ1 elements in *scid* thymocytes (36). The present studies show that signal junction formation for Dδ1-Dδ2 and Dδ2-Jδ1 occurs at the same incidence as (and frequently at a higher incidence than) in phenotypically similar wild-type thymocytes of 15-day fetuses. In contrast to the signal junctions formed by transiently transfected recombination constructs in *scid* transformed lymphoid cell lines, endogenous rearrangements of nontransformed *scid* thymocytes are indistinguishable from wild-type rearrangements. The present data also show that TCR δ signal junctions show age-associated modification, principally by nontemplated nucleotide addition, and that the *scid* mutation does not affect imprecise signal junction resolution.

Although junctional modification of signal joints has been noted to occur at a low frequency, signal ends are generally unmodified prior to ligation (30, 31). In the present study, insertion of nontemplated nucleotides into cloned and sequenced Dδ2-Jδ1 signal junctions was seen in thymocytes from adult but not newborn mice. Thus, nontemplated addition to TCR Dδ2-Jδ1 signal junctions, apparently mediated by terminal deoxynucleotidyltransferase (22), corresponds to age-associated expression of terminal deoxynucleotidyltransferase in thymic ontogeny (37) and to insertion of nontemplated nucleotides into coding junctions (26). Impre-

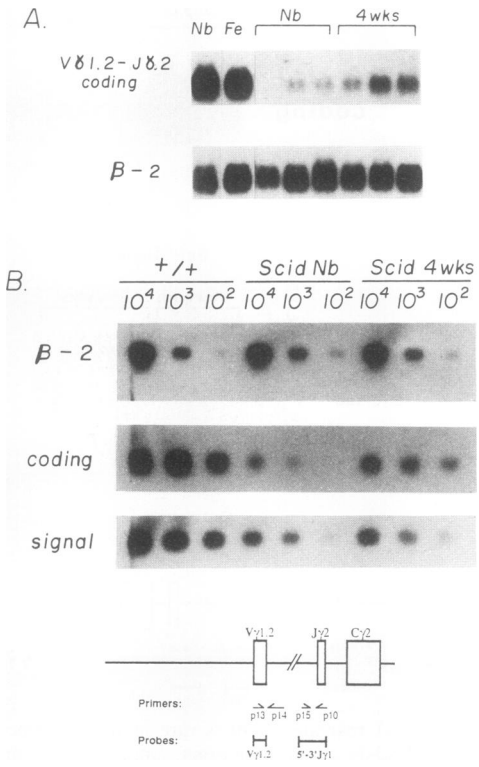

FIG. 7. Vγ1.2-Jγ2 rearrangements are rare in *scid* thymocytes and, when detected, occur in adult (versus newborn) *scid* thymic lysates. (A) PCR-amplified Vγ1.2-Jγ2 coding junctions, amplified with primers p13 and p10, from individual thymic lysates of wild-type (+/+) newborn (Nb) and fetal day 15 (Fe) mice and newborn (Nb) and 4-week-old mice with severe combined immunodeficiency. Hybridization was with the Vγ1.2 probe, detecting an unmodified coding junction of 499 bp. Templates were amplified in parallel with primers for β_2-microglobulin (β-2). (B) Titration of selected templates (thymuses from a wild-type fetal day 15 mouse and newborn and 4-week-old mice with severe combined immunodeficiency) for genome equivalents of 10^4, 10^3, and 10^2, for PCR amplification of Vγ1.2-Jγ2 coding junctions and excised signal junctions (182 bp). Results for a parallel-amplified β_2-microglobulin control (β-2) are also shown. The hybridization probes used were Vγ1.2 (coding) and 5′-3′Jγ1 (signal).

cision for some excised TCR δ signal junctions has been shown (19, 43), and a similar age-associated imprecision of TCR γ signal junctions was recently reported (21). Notably, addition of nontemplated nucleotides to cleaved signal ends in *scid* thymocytes occurs normally. This indicates that biased resolution of signal versus coding ends in *scid* cells is not simply due to the ability to resolve unmodified versus modified strand intermediates.

scid thymic lysates also showed formation of reciprocal inversional Dδ1-Dδ2 hybrid junctions. Although reciprocal hybrid joint formation is detectable for artificial recombination substrates in wild-type cells, the incidence of the reciprocal (inversional) event versus a single hybrid (deletional) event is considerably reduced in frequency (approximately 100-fold) (28). This predicted low incidence (<1%) could account for our failure to detect 5′Dδ1-5′Dδ2 hybrids in wild-type thymocytes. *scid* hybrid-type junctions have been detected in immunoglobulin loci in *scid* Abelson murine leukemia virus-transformed cell lines, within stably integrated recombination cassettes (17), or in endogenous immunoglobulin heavy-chain gene rearrangements (25, 34), in some cases with excessive base loss (17). They have also been detected within transiently transfected substrates (15, 30), and in these studies coding and signal ends showed excessive nucleotide deletion (30). In contrast, *scid* thymocyte hybrid junctions showed no excessive base loss for signal or coding ends. These results indicate that in nontransformed *scid* thymocytes, interaction with a signal end is sufficient to enable normal resolution of a coding end. Similar to signal joint formation, excessive base loss for both coding and signal ends is likely to be exaggerated by the artificial recombination substrate and perhaps by the transformation phenotype as well. The actual incidence of hybrid joint formation in *scid* relative to wild-type thymocytes is not known, but their invariable detection in *scid* thymic lysates may be due to an increased ratio of completed hybrid versus coding joints in *scid* cells.

The present findings that Vδ1-Dδ2, Vγ3-Jγ1, and Vγ1.2-Jγ2 signal junctions are not readily amplifiable in *scid* thymic lysates confirm the TCR element specificity of *scid* thymocyte V(D)J recombinase activity. Unlike Dδ2-Jδ1 and Dδ1-Dδ2 signal junctions, whose incidence was up to 10-fold higher for *scid* than for wild-type thymic lysates, *scid* Vδ- and Vγ-associated signal joints, when detectable, were generally 10-fold lower in incidence. Further, we did not find any evidence of cleaved, unresolved Vδ1 coding ends by Southern analysis.

The basis for this biased TCR δ element recombination in *scid* thymocytes remains unclear. Examination of recombination signal sequences flanking the relevant elements does not indicate that deviation from consensus sequence is a factor. One possible explanation is that the *scid* mutation itself directly or indirectly imposes a bias for recombination of specific elements, for example based on distance between elements or specifically involving V elements. The latter possibility is supported by data of Ferrier et al. (12), who found that transgenic TCR β D-J and V-D gene recombination was differentially regulated in T- and B-lineage cells. Alternatively, the locus and element bias of *scid* V(D)J recombinase activity may mirror a normal ordered, regulated gene rearrangement, possibly for a specific subset, or ontogenic wave, of thymocytes. In this case, Vδ recombination would be regulated similar to immunoglobulin heavy-chain and TCR β loci, in which D-to-J-element-associated recombination precedes V element recombination (2). Within a regulated model, failure to initiate the recombination of V elements could be due to a low incidence of productive D or J δ-associated rearrangements or to progressive alteration of recombinase activity in *scid* cells, as suggested previously (7). TCR δ is probably the first TCR locus to recombine in thymic ontogeny, but ordered recombination of elements within the TCR δ locus has been considered unlikely. This is due to evidence for V-D rearrangement within normal fetal TCR δ alleles which maintain Jδ in the germ line configuration (10). However, specific information about the incidence and timing of TCR δ element recombination for both TCR δ alleles, within clonal cell lineages, is not known, and this explanation remains viable. The detectability of PCR-amplified immunoglobulin heavy-chain gene D-J coding and signal junctions from *scid* bone marrow cells and their normal junctional sequences (44) support this interpretation.

The low levels of detectable Vδ1- and Vγ-associated signal and coding joint formation in individual *scid* thymic lysates are in contrast with conclusions of Kienker et al. (23, 24) that Vδ1 and Vγ elements show ongoing recombination in *scid*

thymocytes. Their findings were based on nonquantitative PCR amplification and cloning of coding junctions from pools of multiple adult thymuses from mice with severe combined immunodeficiency. Also, sequences reported as independent, yet containing identical junctions (eg., Vδ1- and Vγ3-associated coding joints), may have been derived from a very restricted number of DNA templates. Although we have not looked at additional Vδ or Vγ elements, our results show that recombination of Vδ1, Vγ3, and Vγ1.2 is generally 1 to 2 orders of magnitude lower than for Dδ and Jδ1 elements. These V element rearrangements may represent rearrangements within phenotypically distinct thymic populations. These could include "leaky" cells (6) which have undergone phenotypic reversion of the *scid* mutation (35) or cells with normal D-Jδ-associated rearrangements which have progressed to the next stage of ordered recombination, as discussed above.

The present data confirm that aberrancies of the *scid* V(D)J recombinase activity are exaggerated in studies based on transient transfection of artificial recombination substrates into transformed *scid* lymphoid cells. In vivo-generated signal junctions, as well as both signal and coding ends of in vivo-generated hybrid junctions, are indistinguishable from normal ones. Over 50% of *scid* signal junctions in the transient transfection studies showed extensive nucleotide loss, commonly of entire heptamer sequences (30). This difference is probably due to the nonintegrated versus chromosomally integrated recombination substrates; precise signal junctions have been detected in *scid* Abelson murine leukemia virus-transformed cell lines which contain stably integrated recombination substrates (3, 17). Additional distortion of *scid* activity by the transformation phenotype also cannot be ruled out. The present results suggest that the incidence of coding joint formation estimated for transformed *scid* cell lines (1/5,000 that of wild type cells [15]) may be similarly exaggerated. This could account for apparently higher incidences of detectable TCR δ D- and J-element-associated coding junctions (7; data not shown). Further quantitative and qualitative study of in vivo-mediated antigen receptor gene rearrangements within nontransformed lymphocytes is important to the determination of how the *scid* mutation causes developmental arrest in vivo.

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